Isolation of *Leptospira borgpetersenii* in synanthropic *Didelphis albiventris* in Jaboticabal, São Paulo, Brazil

Isolamento de Leptospira borgpetersenii *em* Didelphis albiventris *sinantrópicos em Jaboticabal, São Paulo, Brasil*

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Abstract

Leptospirosis is a waterborne disease and, therefore, stands out for the possibility of environmental contamination, the cross transmission between domestic and wild animals and humans. Opossum species are important reservoirs of this disease making them potential pathogen spreaders. Aiming to verify the presence of *Leptospira* spp. and the antibodies against *Leptospira* spp. in the Campus of São Paulo State University, in Jaboticabal, São Paulo, Brazil, free-living wild life opossum (*Didelphis albiventris*) were captured for blood and urine sampling. Serological analysis was performed Microscopic Agglutination Test (MAT). Aliquots of urine were seeded in media Ellinghausen-McCullough-Johnson-Harris (EMJH) and Fletcher without antibiotics. The samples in which there was growth of leptospires were forwarded to the Leptospirosis Laboratory of the Institute of Pathobiology in the National Institute of Agricultural Technology, Buenos Aires, Argentina and were genotyped using Multiple Locus Variable number tandem repeat Analysis (MLVA). Of the 15 analyzed animals, nine (60.0%) were reactant to Patoc serovar. The pathogenic specie *Leptospira borgpetersenii* was isolated and identified in three *Didelphis albiventris.* The isolation findings of pathogenic specie *Leptopsira borgpetersenii* in the urine culture of three *Didelphis albiventris* in a university campus are a major discovery in the area of preventive veterinary medicine and public health and open a discussion about the important role of free-living wild animals as reservoirs of this agent to domestic animals and humans, a condition that serves as a warning for the improvement of health practices.

Keywords: Isolation. *Leptospira* spp. Brazilian marsupials. MLVA.

Resumo

A leptospirose é uma zoonose de veiculação hídrica e, portanto, se destaca pela possibilidade de contaminação ambiental, o que facilita a transmissão cruzada entre animais domésticos, selvagens e humanos. Espécies de gambás são importantes reservatórios dessa enfermidade, tornando-os potenciais disseminadores do agente. Com o objetivo de verificar a presença de *Leptospira* spp. e de anticorpos contra *Leptospira* spp. no Campus da Universidade Estadual Paulista, em Jaboticabal, foram capturados gambás (*Didelphis albiventris*) de vida livre para a colheita de amostras de sangue e de urina. As análises sorológicas foram efetuadas pela técnica de Soroaglutinação Microscópica (SAM). Alíquotas de urina foram semeadas nos meios Ellinghausen-McCullough-Johnson-Harris (EMJH) e Fletcher sem antibióticos. As amostras que apresentaram crescimento de espiroquetas foram levadas ao Laboratório de Leptospirose do Instituto de Patobiologia, no Instituto Nacional de Tecnologia Agropecuaria, Buenos Aires, Argentina e foram genotipadas com a técnica de Múltiplos Locus de Números Variáveis de Repetição em Tandem (MLVA). Dos 15 animais examinados pela SAM, nove (60,0%) foram reagentes à sorovariedade Patoc. Foi isolada e identificada a espécie patogênica *Leptospira bosrpetersenii* de três *Didelphis albiventris.* Os achados de isolamento da espécie patogênica *Leptospira borgpetersenii* na cultura de urina de três *Didelphis albiventris* são um grande descobrimento para as áreas da medicina veterinária preventiva e da saúde pública e reforçam a discussão sobre o importante papel dos animais selvagens de vida livre como reservatórios desse agente para animais domésticos e seres humanos, situação que serve de alerta para melhorias nas práticas sanitárias.

Palavras-chave: Isolamento. *Leptospira* spp. Marsupiais brasileiros. MLVA.

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Introduction

Leptospirosis is an infection disease. Its occurrence is directly related to poor sanitary conditions, which are frequently seen in different Brazilian regions (BRASIL, 2005). Floods favors the dissemination and the persistence of the causal agent in the environment, predisposing outbreaks, once rainwater and river water mix with wastewater usually contaminated by infected animal urine (BRASIL, 2005).

Opossums are very adaptable animals with respect to their great variety of living habitat. Because of this huge environmental flexibility, they may bring losses to public health, mainly because they are reservoirs of many zoonotic potential diseases. Reilly, Ferris and Hanson (1968) and Michna and Campbel (1970) reported that rodents, marsupials, xenarthra, carnivores and artiodactyls can be important sources of leptospirosis infection. Among marsupials of didelphimorphia order, the opossums (*Didelphis marsupialis* and *Didelphis virginianus*) were described with antibodies titers against serovars Ballum, Bataviae, Icterohaemorrhagiae, Szwajizam and Grippotyphosa (SANTA ROSA et al., 1975; CALDAS, FEHRINGER; SAMPAIO, 1992; HATHAWAY et al., 1981), suggesting the important role of these species as reservoirs of leptospires (DUHAMEL et al., 1998). Jorge et al. (2012) reported that Didelphidae families are considered susceptible to infection by a wide range of *Leptospira* serovars and the species serve as reservoirs of this agent.

Brazil is a country of large longitudinal extension (different climates and vegetation formation), constituting a large biodiversity environment, with an intense proximity between wild animals, domestic animals and humans and due to the endemism, the occurrence of leptospirosis. Thus, the aim of this study was to verify the occurrence of *Leptospira* spp. and seroreactivity to *Leptospira* spp. in free-living *Didelphis albiventris* of the Campus of São Paulo State University in Jaboticabal, São Paulo, Brazil.

Methodology

Between April and June 2011, using tomahawk traps baited with sausage and bologna, 15 free living *Didelphis albiventris,* nine females and six males, all adults, were captured from different regions of the Campus of São Paulo State University in Jaboticabal, São Paulo. For the present study, authorization was granted from the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) to work with free living wild animals, concession number 26185-1 (02/07/2011 at 16:18 h). Authorization was also granted from Comissão de Ética no Uso de Animais (CEUA), to work with wild and domestic animals, protocol number 027958/10 (12/20/2010). This document also states that the present research is consistent with the Princípios Éticos na Experimentação Animal, adopted by the Colégio Brasileiro de Experimentação Animal (COBEA).

The captured animals were first physically restrained using leather gloves and, after immobilization, anesthetized with 0,1mL/Kg of zoletil^{R} intramuscularly in the lateral femoral region.

After complete sedation, vital signs were measured: temperature, heart rate, respiratory rate, examination of mucosal color and palpebral, podal and caudal reflexes.

After local antisepsis, 2mL of blood was collected from the caudal vein, utilizing sterile and disposable syringes and needles. The blood samples were placed in sterile 5mL hemolysis type tubes, and forwarded to the Brucellosis and Leptospirosis Diagnosis Laboratory of Preventive Veterinary Medicine and Animal Reproduction Department of São Paulo State University in Jaboticabal.

Urine samples were then collected by cystocentesis or, in some cases, during spontaneous urinating induced by the anesthesia. A 0.22 μm filter was coupled to the syringe tip, and near the Bunsen burner, simultaneously one drop of filtered urine was

transferred to a sterile tube with liquid culture media Ellinghausen-McCullough-Johnson-Harris (EMJH) without antibiotics and one drop in a sterile tube with semisolid culture media Fletcher (THIERMANN, 1980; ELLIS et al., 1982). These tubes were kept in a bacteriological incubator BOD at 28°C. At the end of the procedure, collection areas were sanitized with iodine and the animal was put inside the trap until complete post-sedation recovery. Afterwards, each animal was released in the same capture site.

Tubes with blood samples were centrifuged at 5000G for obtaining the blood serum. Serum aliquots were then transferred to 2mL plastic tubes and kept under freezing temperatures (-20°C) until the examination time. For the leptospirosis, serologic diagnostic was performed using Microscopic Agglutination Test (MAT), as recommended by OIE (2012).

Serovars Australis, Bratislava, Autumnalis, Butembo, Castellonis, Bataviae, Canicola, Whitcombi, Cynopteri, Grippotyphosa, Hebdomadis, Copenhageni, Icterohaemorrhagiae, Javanica, Panama, Pomona, Pyrogenes, Hardjo, Wolffi, Shermani, Tarassovi, Andamana, Patoc and Sentot were used as antigen. All antigens were pure and used around the sixth incubation day. The concentration considered optimal was standardized to correspond to half turbidity of the tube number 1 in MacFarland scale (about 100 to 200 leptospires per microscopic field), according to the Sulzer and Jones (1980) technique. The cultures were free from contamination and autoagglutination.

The presence of antibodies against *Leptospira* spp. in the serum was verified by MAT according to OIE (2012) and the criterion adopted to consider a serum as reactant was the agglutination of at least 50% of leptospiras in the microscopic field in the 100x rise. The reactant serum in the screening were reexamined in four serial dilution of reason two and only the samples with minimum title of 100 (cut point) were selected (SANTA ROSA et al., 1975, 1980).

Urine cultures were evaluated weekly during a period variable from eight to sixteen weeks (THIERMANN, 1980; ELLIS et al., 1982). When the *Leptospira* spp. growing ring were observed in Fletcher medium, the culture was subcultured in modified EMJH medium without antibiotics (FAINE, 1999). The tubes that showed contamination in the weekly evaluation were discarded. From the EMJH medium with *Leptospira* spp. growth and without contamination an aliquot were taken to darkfield microscopy observation.

The samples in which there was growth of leptospires were forwarded to the Leptospirosis Laboratory of the Institute of Pathobiology in the National Institute of Agricultural Technology, Buenos Aires, Argentina and were genotyped using Multiple Locus Variable number tandem repeat Analysis (MLVA). MLVA strain typing procedure was performed using primers flanking the VNTRs: 4, 7, 9, 10, 19, 23, 31, Lb4 and Lb5 were used to discriminate strains of *L. Interrogans* and *L. borgpetersenii* (PAVAN et al., 2011).

Results

Of the 15 analyzed animals, nine (60.0%) were reactant to Patoc serovar. From the nine females, five (55.6%) were reactant, as were four of the six males (66.7%) (Table 1).

The pathogenic specie *Leptospira borgpetersenii* was isolated by urine culture and identified by MLVA in three of 15 *Didelphis albiventris* analyzed, two females and one male (Table 1 and Figure 1).

Discussion

Nothing relevant can be concluded from the results of serology, since the only serovar against antibody titers found in MAT was Patoc, which is a saprophytic species of *Leptospira* spp. A similar situation was emphasized by Yasuda et al. (1986), in which the authors stated the impossibility of completing that a saprophytic leptospira infection as *Leptospira biflexa* could be the cause of abortions. Still, as cited by Myers (1976), the occurrence of cross-reactivity of a saprophytic with antibodies induced by a pathogenic

Table 1 - Results of microscopic agglutination test to leptospirosis and samples of urine for isolation findings of *Leptospira* spp. in each one of the 15 free-living *Didelphis albiventris* examined in the Campus of São Paulo State University. Jaboticabal – São Paulo, Brazil – 2013

Specimen	Gender	Category	Result	Serovar	Antibody titer	Isolation
1	Male	Adult	Positive	Patoc	800	-
2	Female	Adult	Negative	-	-	-
3	Male	Adult	Negative	-	-	-
4	Female	Adult	Positive	Patoc	100	-
5	Female	Adult	Positive	Patoc	400	*Leptospira borgpetersenii*
6	Female	Adult	Positive	Patoc	100	-
7	Male	Adult	Negative	-	-	-
8	Male	Adult	Positive	Patoc	200	-
9	Male	Adult	Positive	Patoc	800	-
10	Female	Adult	Negative	-	-	-
11	Female	Adult	Positive	Patoc	400	-
12	Female	Adult	Positive	Patoc	800	*Leptospira borgpetersenii*
13	Female	Adult	Negative	-	-	-
14	Female	Adult	Negative	-	-	-
15	Male	Adult	Positive	Patoc	800	*Leptospira borgpetersenii*

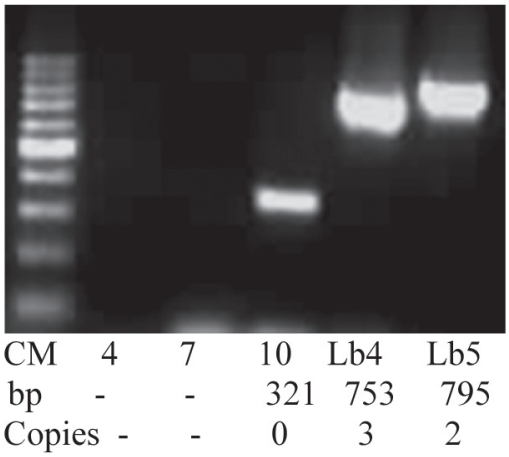

Figure 1 - A photo of gel regarding the *Leptospira borgpetersenii* isolated from urine culture of three females *Didelphis albiventris* captured in the Campus of São Paulo State University - Jaboticabal, São Paulo, Brazil – 2013

Source: acervo S. G. Loffler

serovar not included in the battery of antigens used in MAT in this study. On the other hand, Vasconcellos et al. (1989) described the absence of humoral immune response of pigs that received drinking water contaminated with *L. biflexa* strain Buenos Aires, found that this strain was not able to infect swine by the oral route in natural conditions.

The present study isolated by urine cultures and identified by MLVA the pathogenic specie *Leptospira borgpetersenii* in three *Didelphis albiventris.* Successful isolation results from Leptospira spp. were also obtained by Jorge et al. (2012), who isolated and identified *Leptospira borgpetersenii* serovar Castellonis through the cultivation of urine of thirty-three free-living *Didelphis albiventris*, and by Felix et al. (2008), who isolated strains of *Leptospira borgpetersenii* serogroup Ballum in four mice.

Poor sanitary practices, such as buildup of debris and organic waste, lack of sanitation of animal feeders and drink dispensers, poor packaging of feed and neglect of water quality favor the spread of synanthropic organisms such as mice, opossums and guinea pigs in urbanized locales and, thus, narrows the contact between these potential reservoirs of pathogenic species of *Leptospira* spp. and people and their pets.

The isolation findings of pathogenic specie *Leptospira borgpetersenii* in the urine cultures of three *Didelphis albiventris* in a university campus are a major discovery in the area of preventive veterinary medicine and public health and reinforce the discussion about the important role of free-living wild

animals as reservoirs of this agent to domestic animals and humans, a situation that serves as a warning for the improvement of health practices.

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